(i) Its potency is not less than 600 micrograms of neomycin per milligram, calculated on an anhydrous basis.

(ii) [Reserved]

(iii) Its loss on drying is not more than 8.0 percent.

(iv) Its pH in an aqueous solution containing 33 milligrams per milliliter is not less than 5.0 and not more than 7.5.

(v) It gives a positive identity test for neomycin.

(2) *Labeling.* It shall be labeled in accordance with the requirements of § 432.5 of this chapter.

(3) *Requests for certification; samples.* In addition to complying with the requirements of § 431.1 of this chapter, each such request shall contain:

(i) Results of tests and assays on the batch for potency, loss on drying, pH, and identity.

(ii) Samples required: 10 packages, each containing approximately 300 milligrams.

(b) *Tests and methods of assay*—(1) *Potency.* Proceed as directed in § 436.105 of this chapter, preparing the sample for assay as follows: Dissolve an accurately weighed sample in sufficient 0.1*M* potassium phosphate buffer, pH 8.0 (solution 3), to give a stock solution of convenient concentration. Further dilute an aliquot of the stock solution with solution 3 to the reference concentration of 1.0 microgram of neomycin per milliliter (estimated).

(2) [Reserved]

(3) *Loss on drying.* Proceed as directed in § 436.200(a) of this chapter.

(4) *pH.* Proceed as directed in § 436.202 of this chapter, using a solution containing 33 milligrams of neomycin per milliliter.

(5) *Identity*—(i) *Reagents.* (*a*) Sulfuric acid solution: Mix concentrated sulfuric acid and distilled water in volumetric proportions of 40:60.

(*b*) Xylene.

(*c*) *p*-Bromoaniline: (Prepare and store this reagent in brown, nonactinic glassware.) Place 380 milliliters of thiourea-saturated glacial acetic acid solution in the bottle, add 10 milliliters of 20 percent sodium chloride solution, 5 milliliters of 5 percent oxalic acid solution, and 5 milliliters of 10 percent disodium phosphate solution, and mix well. Add 8 grams of *p*-bromoaniline and mix well. Let this reagent stand overnight before use. Prepare the reagent once weekly.

(ii) *Procedure.* Place about 10 milligrams of the sample into a test tube (19 millimeters × 150 millimeters), dissolve with 1 milliliter of water, and then carefully add 5 milliliters of the sulfuric acid solution. Heat in a boiling water bath for 100 minutes. Cool to room temperature. Add 10 milliliters of xylene to the test tube. Stopper the tube and shake vigorously for about 1 minute. Let the two layers separate and then decant the xylene layer into a second test tube. Add 10 milliliters of the *p*-bromoaniline reagent to the xylene solution, shake, and let stand. The development of a vivid pink-red color is a positive identity test for neomycin.

[40 FR 22252, May 22, 1975, as amended at 50 FR 19919, May 13, 1985]

§ 444.42a Sterile neomycin sulfate.

(a) *Requirements for certification*—(1) *Standards of identity, strength, quality, and purity.* Neomycin sulfate is the sulfate salt of a kind of neomycin or a mixture of two or more such salts. It is so purified and dried that:

(i) It has a potency of not less than 600 micrograms of neomycin per milligram, calculated on an anhydrous basis.

(ii) It is sterile.

(iii) It is nonpyrogenic.

(iv) [Reserved]

(v) Its moisture content is not more than 8.0 percent.

(vi) Its pH in an aqueous solution containing 33 milligrams per milliliter is not less than 5.0 and not more than 7.5.

(vii) It gives a positive identity test for neomycin.

(2) *Labeling.* It is to be labeled in accordance with the requirements of § 432.5(b) of this chapter.

(3) *Request for certification; samples.* In addition to the requirements of § 431.1 of this chapter, each such request shall contain:

(i) Results of tests and assays on the batch for potency, sterility, pyrogens, moisture, pH, and identity.

(ii) Samples required;

(*a*) For all tests except sterility: 10 packages, each containing approximately 300 milligrams.

(*b*) For sterility testing: 20 packages, each containing approximately 300 milligrams.

(b) *Tests and methods of assay*—(1) *Potency*. Use either of the following methods:

(i) *Plate assay using Staphylococcus epidemmidis (ATCC 12228)*[1]—(*a*) *Cylinders (cups)*. Use cylinders described in § 440.80a(b)(1)(i) of this chapter.

(*b*) *Culture media.* Using ingredients that conform to the standards prescribed by the U.S.P. or N.F.:

(*1*) Make nutrient agar for carrying the test organism as follows:

Peptone	6.0 gm.
Pancreatic digest of casein	4.0 gm.
Yeast extract	3.0 gm.
Beef extract	1.5 gm.
Dextrose	1.0 gm.
Agar	15.0 gm.
Distilled water q.s	1,000.0 ml.

pH 6.5 to 6.6 after sterilization.

(*2*) Make nutrient agar for the base and seed layers as described in paragraph (b)(1)(i)(*b*)(*1*) of this section, except that its pH after sterilization is 7.8 to 8.0.

In lieu of preparing the media from the individual ingredients specified in paragraph (b)(1)(i)(*b*) of this section, they may be made from a dehydrated mixture that, when reconstituted with distilled water, has the same composition as such media. Minor modification of the individual ingredients specified in paragraph (b)(1)(i)(*b*) of this section are permissible if the resulting media possess growth-promoting properties at least equal to the media described.

(*c*) *Working standard.* Dry a portion of the working standard for 3 hours at 60° C. and a pressure of 5 millimeters or less. Determine the dry weight, and dissolve in sufficient 0.1*M* potassium phosphate buffer, pH 8.0, to give a stock solution of convenient concentration. When stored under refrigeration, the stock solution may be used for a period not exceeding 2 weeks.

(*d*) *Preparation of sample.* Dissolve an accurately weighed sample in sufficient 0.1*M* potassium phosphate buffer, pH 8.0 (solution 3), to give a stock solution of convenient concentration. Further dilute the stock solution with sufficient solution 3 to obtain a reference concentration of 1.0 microgram of neomycin per milliliter (estimated).

(*e*) *Preparation of test organism.* The test organism is *Staphylococcus epidermidis* (ATCC 12228),[1] which is maintained on slants of nutrient agar described in (b)(1)(i)(*b*)(*1*) of this section. Using 3 milliliters of U.S.P. saline T.S., wash the organism from the nutrient agar slant (which has been incubated for 24 hours at 32° C.−35° C.) onto a large nutrient agar surface such as that provided by a Roux bottle containing 300 milliliters of nutrient agar. Incubate for 24 hours at 32° C.−35° C. Wash the resulting growth from the nutrient surface, using 50 milliliters of sterile U.S.P. saline T.S. Adjust the volume of the suspension so that a 1:14 dilution will give 25 percent light transmission when measured with a suitable photo-electric colorimeter having a 580 mμ filter and a 13-millimeter diameter test tube as an absorption cell. By the use of test plates, determine the appropriate inoculum of the adjusted suspension (usually 0.1 milliliter) to be inoculated to each 100 milliliters of seed layer agar in order to obtain satisfactory zones of inhibition. The suspension may be used for 1 week if stored under refrigeration.

(*f*) *Preparation of plates.* Add 21 milliliters of the agar prepared as described in paragraph (b)(1)(i)(*b*)(*2*) of this section to each Petri dish (20 millimeters × 100 millimeters). Distribute the agar evenly in the plates and allow to harden on a level surface. Accurately measure a sufficient quantity of the nutrient agar, cool to 48° C., and add the appropriate inoculum of the adjusted suspension, prepared as described in paragraph (b)(1)(i)(*e*) of this section. Swirl the inoculated nutrient agar to obtain a homogeneous suspension, and add 4 milliliters to each of the plates containing the 21 milliliters of uninoculated nutrient agar. Tilt the plates back and forth to spread the inoculated nutrient agar evenly, and allow to harden on a level surface. After the agar has hardened, place six cylinders described in paragraph

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

(b)(1)(i)(*a*) of this section on the inoculated agar surface so that they are at approximately 60° intervals on a 2.8-centimeter radius. Use the plates the same day they are prepared.

(*g*) *Standard curve.* Using the stock solution of the working standard prepared as described in paragraph (b)(1)(i)(*c*) of this section, prepare solutions in 0.1*M* potassium phosphate buffer pH 8.0 of the following concentrations: 0.64, 0.8, 1.0, 1.25, 1.56 micrograms of neomycin per milliliter. The 1.0 microgram per milliliter concentration is the reference concentration of the assay. Use a total of 12 plates, three plates for each solution except the reference point solution which is included on each plate. On each of the three plates, fill three cylinders with the reference point solution and the other three cylinders with the concentrations under test. Thus, there will be 36 reference point determinations and nine determinations for each of the other points on the curve. After the plates have incubated, read the diameters of the circles of inhibition. Average the readings of the reference point concentration and the readings of the point tested for each set of three plates and average also all 36 readings of the reference point concentration. The average of the 36 readings of the reference point concentration is the correction point of the curve. Correct the average value obtained for each point to the figure it would be if the reference point reading for that set of three plates were the same as the correction point. Thus, if in correcting the 0.8-microgram concentration, the average of the 36 readings of the 1.0 microgram per milliliter (reference point) concentration is 16.5 millimeters and the average of the 1.0 microgram per milliliter concentration of the set of three plates (the 0.8 microgram per milliliter set) is 16.3 millimeters, the correction is +0.2 millimeter. If the average readings of the 0.8 microgram per milliliter concentration of these same three plates is 15.9 millimeters, the corrected value is then 16.1 millimeters. Plot these corrected values, including the average of the 1.0 microgram per milliliter concentration, on 2-cycle semilog paper, using the concentration in micrograms per milliliter as the ordinate (the logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points either by inspection or by means of the following equations:

$$H=(3a+2b+c-\mathrm{e})/(5),$$
$$L=(3e+2d+\mathrm{c}-\mathrm{a})/(5),$$

where:

L=Calculated zone diameter for the lowest concentration of the standard curve;
H=Calculated zone diameter for the highest concentration of the standard curve;
c=Average zone diameter of 36 readings of the reference point standard solution;
a, b, d, e=Corrected average values for the other standard solutions, lowest to highest concentrations, respectively.

(*h*) *Assay procedure.* Use three plates for each sample. Fill three cylinders on each plate with the standard and three cylinders with the sample, which has been diluted to the reference concentration, alternating standard and sample. Incubate the plates for 16 hours to 18 hours at 32° C.–35° C., and then measure the diameter of each zone of inhibition. To estimate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on the three plates used. If the sample gives a larger zone size than the average of the standard, add the difference between them to the reference point zone of the standard curve. If the average value is lower than the standard value, subtract the difference between them from the reference point value on the curve. From the curve, read the potencies corresponding to these corrected values of zone sizes.

(ii) *Plate assay using Staphylococcus aureus (ATCC 6538P).*[1] Proceed as directed in paragraph (b)(1)(i) of this section except that the reference concentration of the sample under test is 10.0 micrograms of neomycin per milliliter; the concentrations of the standard curve solutions are 6.4, 8.0, 10.0, 12.5, 15.6 micrograms of neomycin per milliliter; and the suspension of the test organism, staphylococcus aureus (ATCC 6538P),[1] is adjusted so that a 1:19

[1] Available from: American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852.

dilution will give 25 percent light transmission and the usual inoculum for each 100 milliliters of agar for the seed layer is 0.2 milliliter of diluted suspension.

(2) *Sterility.* Proceed as directed in § 436.20 of this chapter, using the method described in paragraph (e)(1) of that section.

(3) *Pyrogens.* Proceed as directed in § 440.80a(b)(3) of this chapter, using a test dose of 1.0 milliliter per kilogram of a solution containing 10 milligrams of neomycin per milliliter in pyrogen-free, sterile U.S.P. saline T.S.

(4) [Reserved]

(5) *Moisture.* In an atmosphere of about 10 percent relative humidity, transfer about 100 milligrams of the finely powdered sample to a tared weighing bottle equipped with ground-glass top and stopper. Weigh the bottle and place it in a vacuum oven, tilting the stopper on its side so that there is no closure during the drying period. Dry at a temperature of 60° C. and a pressure of 5 millimeters of mercury or less for 3 hours. At the end of the drying period, fill the vacuum oven with air dried by passing it through a drying agent such as sulfuric acid or silica gel. Replace the stopper and place the weighing bottle in a desiccator over a desiccating agent such as phosphorus pentoxide or silica gel, allow to cool to room temperature, and reweigh. Calculate the percent loss.

(6) *pH.* Proceed as directed in § 440.80a(b)(5)(ii) of this chapter, using a solution containing 33 milligrams of neomycin per milliliter.

(7) *Identity*—(i) *Reagents.* (*a*) Sulfuric acid solution: Mix concentrated sulfuric acid and distilled water in volumetric proportions of 40:60.

(*b*) Xylene.

(*c*) *p*-Bromoaniline: (Prepare and store this reagent in brown, nonactinic glassware.) Place 380 milliliters of thioureasaturated glacial acetic acid solution in the bottle, add 10 milliliters of 20 percent sodium chloride solution, 5 milliliters of 5 percent oxalic acid solution, and 5 milliliters of 10 percent disodium phosphate solution, and mix well. Add 8 grams of *p*-bromoaniline and mix well. Let this reagent stand overnight before use. Prepare the reagent once weekly.

(ii) *Procedure.* Place about 10 milligrams of the sample into a test tube (19 millimeters × 150 millimeters), dissolve with 1 milliliter of water, and then carefully add 5 milliliters of the sulfuric acid solution. Heat in a boiling water bath for 100 minutes. Cool to room temperature. Add 10 milliliters of xylene to the test tube. Stopper the tube and shake vigorously for about 1 minute. Let the two layers separate and then decant the xylene layer into a second test tube. Add 10 milliliters of the *p*-bromoaniline reagent to the xylene solution, shake, and let stand. The development of a vivid pink-red color is a positive identity test for neomycin.

[39 FR 19046, May 30, 1974, as amended at 50 FR 19919, May 13, 1985; 53 FR 12660, Apr. 15, 1988]

§ 444.46 Netilmicin sulfate.

(a) *Requirements for certification*—(1) *Standards of identity, strength, quality, and purity.* Netilmicin sulfate is the sulfate salt of D-Streptamine,4-*O*-[3-amino-6-(aminomethyl)-3,4-dihydro-2*H*-pyran-2-yl]-2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-N^1-ethyl-, (2*S*-*cis*)-, (2:5). It is a white-to-buff-colored powder. It is so purified and dried that:

(i) Its potency is not less than 595 micrograms of netilmicin per milligram on an anhydrous basis.

(ii) Its loss on drying is not more than 15.0 percent.

(iii) Its pH in an aqueous solution containing 40 milligrams per milliliter is not less than 3.5 and not more than 5.5.

(iv) Its residue on ignition is not more than 1.0 percent.

(v) Its specific rotation in an aqueous solution containing 30 milligrams per milliliter at 25° C is not less than +88° and not more than +96°.

(vi) It passes the identity test.

(2) *Labeling.* It shall be labeled in accordance with the requirements of § 432.5 of this chapter.

(3) *Requests for certification; samples.* In addition to complying with the requirements of § 431.1 of this chapter, each such request shall contain:

(i) Results of tests and assays on the batch for potency, loss on drying, pH,